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BACKGROUND. To reduce the treatment burden for patients with neovascular age-related macular degeneration (nvAMD), emerging therapies targeting vascular endothelial growth factor (VEGF) are being designed to extend the interval between treatments, thereby minimizing the number of intraocular injections. However, which patients will benefit from longer-acting agents is not clear.

METHODS. Eyes with nvAMD (n=122) underwent 3 consecutive monthly injections with currently available anti-VEGF therapies, followed by a treat-and-extend protocol. Patients who remained quiescent 12 weeks from their prior treatment entered a “treatment pause” and were switched to pro re nata (PRN) treatment (based on vision, clinical exam, and/or imaging studies). Proteomic analysis was performed on aqueous fluid to identify proteins that correlate with patients’ response to treatment.

RESULTS. At the end of 1 year, 38/122 eyes (31%) entered a treatment pause (≥30 weeks). Conversely, 21/122 eyes (17%) failed extension and required monthly treatment at the end of year 1. Proteomic analysis of aqueous fluid identified proteins that correlated with patients’ response to treatment including proteins previously implicated in AMD pathogenesis. Interestingly, apolipoprotein-B100 (ApoB100), a principal component of drusen implicated in the progression of non-neovascular AMD, was increased in treated patients […]
Aqueous Proteins Help Predict the Response of Neovascular Age-related Macular Degeneration Patients to Anti-VEGF Therapy

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Funding: This work was supported by the National Eye Institute, National Institutes of Health grants R01EY029750 to A.S. and J.Q., R01EY025705 to S.M. and A.S., and R01 EY27961 to J.T.H.; the Research to Prevent Blindness, Inc., Special Scholar Award and an unrestricted grant to A.S.; the Alcon Young Investigator Award from the Alcon Research Institute to A.S., the Robert Bond Welch Professorship to J.T.H.; and the Branna and Irving Sisenwein Professorship in Ophthalmology to A.S. The funding organizations had no role in the design or conduct of this research.

Authors' Contributions: AS is the primary contributor to research design. XC, JCS, AD, CG, TPP, ZY, LC, YW, DM, KJ, YD, and AS are responsible for research execution and data acquisition. XC, JCS, AD, CG, TPP, M-WH, JQ, HZ, SM, and AS are the primary contributors to data analysis and interpretation. Manuscript preparation by AS with revisions provided by XC, JCS, M-WH, JTH, JQ, HZ, and SM.

Running Title: Predicting the response of nvAMD patients to anti-VEGF therapy

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Key Words: Age-related macular degeneration (AMD), Neovascular AMD (nvAMD), Non-neovascular AMD (nnvAMD), Vascular endothelial growth factor (VEGF), anti-VEGF therapy, choroidal neovascularization, treat-and-extend, pro re nata, proteomics, complement, complement factor 3 (C3), complement factor H (CFH), complement factor H-related (CHFR) proteins, apolipoprotein-B100 (ApoB100), drusen
Abstract

Background: To reduce the treatment burden for patients with neovascular age-related macular degeneration (nvAMD), emerging therapies targeting vascular endothelial growth factor (VEGF) are being designed to extend the interval between treatments, thereby minimizing the number of intraocular injections. However, which patients will benefit from longer-acting agents is not clear.

Methods: Eyes with nvAMD (n=122) underwent 3 consecutive monthly injections with currently available anti-VEGF therapies, followed by a treat-and-extend protocol. Patients who remained quiescent 12 weeks from their prior treatment entered a “treatment pause” and were switched to pro re nata (PRN) treatment (based on vision, clinical exam, and/or imaging studies). Proteomic analysis was performed on aqueous fluid to identify proteins that correlate with patients’ response to treatment.

Results: At the end of 1 year, 38/122 eyes (31%) entered a treatment pause (≥30 weeks). Conversely, 21/122 eyes (17%) failed extension and required monthly treatment at the end of year 1. Proteomic analysis of aqueous fluid identified proteins that correlated with patients’ response to treatment including proteins previously implicated in AMD pathogenesis. Interestingly, apolipoprotein-B100 (ApoB100), a principal component of drusen implicated in the progression of non-neovascular AMD, was increased in treated patients who required less frequent injections. ApoB100 expression was higher in AMD eyes compared to controls but was lower in eyes that develop choroidal neovascularization (CNV), consistent with a protective role. Accordingly, mice over-expressing ApoB100 were partially protected from laser-induced CNV.

Conclusions: Aqueous biomarkers could help identify nvAMD patients who may not require – nor benefit from – long-term treatment with anti-VEGF therapy.

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Introduction

Neovascular (nv) age-related macular degeneration (AMD) is the leading cause of severe vision loss in elderly Americans (1). If left untreated, patients with nvAMD suffer dramatic and irreversible vision loss from edema, bleeding and scarring caused by growth of abnormal leaky blood vessels (i.e., choroidal neovascularization or CNV). The recent introduction of therapies targeting vascular endothelial growth factor (VEGF), has had a remarkable impact on patients with nvAMD (2). VEGF is a potent endothelial mitogen and permeability factor that is regulated by the transcription factor, hypoxia-inducible factor (HIF)-1 (3-5). Several multi-centered, randomized, controlled clinical trials have demonstrated that a minority of nvAMD patients treated with anti-VEGF therapy lose further vision, with up to half experiencing a clinically-significant (i.e., 3 line or more on the ETDRS chart) improvement in vision (6). However, anti-VEGF therapies are provided indefinitely, raising concerns about the substantial economic and social burden of frequent clinic visits for elderly patients who often require assistance for transportation and mobility (7). While this has prompted the development of longer-acting anti-VEGF therapies which require less frequent injections (8), there are also concerns about ocular risks – and theoretical concerns about the safety – of frequent, indefinite intraocular injections with anti-VEGF therapies (9, 10). Given rising health care costs, an aging population, and the anticipated increase in the number of patients with nvAMD worldwide, the sustainability of indefinite intraocular injections with anti-VEGF therapy is a reasonable concern. This concern has prompted some clinicians to seek to reduce the number of treatments with currently available therapies (and, in turn, injection/treatment-related adverse events) and the number of visits. This may be one explanation for why the success of anti-VEGF therapies, when translated to real-world clinical practice, has been less impressive than that observed in clinical trials (11). Cao et al., 3
Investigators are therefore exploring alternative approaches that refine initial treatment protocols without sacrificing the visual acuity benefits observed with monthly or bi-monthly treatment.

One approach many clinicians have adapted to reduce the treatment burden is to monitor patients with nvAMD using a fixed interval, but to only treat patients “as needed” or pro re nata (PRN). This approach has the potential to reduce the total annual number of injections but would not impact either the frequency of patient visits or the number of imaging studies performed. This “reactive” approach presumes to capture most relapses promptly while minimizing the number of treatments. In the CATT study, PRN treatment with ranibizumab was demonstrated to be non-inferior to monthly treatment but reduced the number of injections by more than a third (from 24 to 15) by the end of year two; similar results were observed with PRN treatment with bevacizumab (12). These results were corroborated in the HARBOR study (13). However, with a fixed treatment interval, the number of patient visits was the same in the monthly- and PRN-treated patients.

An alternative approach to optimize the efficacy of a drug for each patient while minimizing the number of injections is to “treat-and-extend” (TAE), in which the response of an individual patient to treatment is used to determine whether the interval between treatments can be extended for that particular patient; this approach provides mandatory dosing but at a personalized schedule. TAE is a “proactive” approach that assumes that patients manifest a regular pattern of disease activity (i.e., a patient’s response to their previous injection can predict their response to a subsequent injection). The TREND study demonstrated that the TAE approach was non-inferior to monthly dosing with ranibizumab and resulted in a decreased mean number of injections by 22% (11.1 vs. 8.7) and visits by 21% (11.2 vs. 8.9) in the first year compared to monthly dosing (14). Similar results were observed in the CANTREAT study which Cao et al., 4
also demonstrated non-inferiority for TAE with ranibizumab compared to monthly treatment and reduced the number of injections by 25% (from 23.5 to 17.6) compared to monthly treatment over two years (15). Both studies also demonstrated that up to a quarter of patients “fail” attempts to extend the interval between treatments, requiring monthly injections. While half of patients in the TRENDD study required treatment every 4-8 weeks despite using the TAE approach, approximately 20% of nvAMD patients could be extended to 12 weeks or more between injections, at which point they received a “maintenance treatment”. TAE therefore can reduce annually both the total number of visits as well as the total number of injections. However, TAE may still result in over-treating patients during the extension phase and may be treating patients unnecessarily during the maintenance phase.

Here we use a hybrid of the PRN and TAE approaches, which we describe as “treat-and-extend-pause/monitor” (TEP/M), to assess whether we can safely and effectively wean nvAMD patients off anti-VEGF therapy. We then categorize patients based on the interval between treatments required to adequately manage their disease by the end of 1 year and performed proteomic analyses of aqueous fluid (obtained at the time of treatment initiation) comparing patients who require monthly treatment with those who required less frequent treatment. Using this unbiased approach, we set out to identify proteins that predict – or directly contribute to – the response of nvAMD patients to treatment with anti-VEGF therapy.
Results

Ascertainment of Study Eyes of Primary Cohort

A review of charts of patients from the clinic of a single vitreoretinal surgeon at a satellite office of a tertiary care center from 2013 to 2020 identified 207 eyes from insured patients with a diagnosis of nvAMD (diagnosed by clinical exam and SD-OCT and confirmed by fluorescein angiography) who underwent intravitreal injections with anti-VEGF therapy (Table 1 and Supplemental Figure 2). Inclusion and exclusion criteria are described in the methods section. Patients included in the study agreed to participate in the TEP/M approach and were followed after initiating treatment without interruption for 1 year (102 eyes; 87 patients) and at 2 years (65 eyes; Table 1 and Supplemental Figure 2).

Outcome measurements at 12 and 24 months during steady state phase of TEP/M protocol.

Following the TEP/M protocol, the mean change in central subfield thickness (CST) on SD-OCT was -79.3 ±9.6 and the mean change in vision was 2 ±2 letters at the end of year 1 (Table 2). The percent of patients with a mean improvement or decline in vision of 5 letters or greater was 46% and 19%, respectively. The mean interval between treatments (using a maximal interval of 6 months for patients who were weaned from treatment) was 11.3 ±0.7 weeks by the end of year 1. Accordingly, the mean number of treatments received was 7.8 ±0.2. Compared to traditional monthly treatment, the number of treatments using the TEP/M protocol was reduced by 40% (from 13.0 to 7.8) at 12 months.

There were 65 eyes that were followed under the TEP/M protocol for at least two years without deviations from protocol, as described for year 1. The mean interval between treatments using a maximal interval of 6 months for patients who were weaned from treatment, was 14.6 months.
±1.1 weeks after 24 months (Table 3). In turn, the mean number of treatments received by the end of year 2 was 12.2 ±1.1. Compared to traditional bi-monthly treatment (following 3 initial monthly treatments), the number of treatments using the TEP/M protocol was reduced by 13% (from 14.0 to 12.2) at 24 months (Table 3). Compared to traditional monthly treatment, the number of treatments using the TEP/M protocol was reduced by 51% (from 25.0 to 12.2) at 24 months.

**Patients successfully weaned from anti-VEGF therapy using TEP/M approach.**

Using the TEP/M protocol, we were able to successfully wean off treatment (i.e., patients not requiring treatment on 3 consecutive scheduled visits, and for at least 30 weeks from their last injection) 31% (32/102) of patients within the first year (Supplemental Table 1; Table 4). By the end of year two, 38% (25/65) of patients were weaned from treatment. Of the 22 patients successfully weaned off treatment who were followed for a minimum of 2 years, 73% (16/22) remained off treatment at the end of year 2 and 87% (13/15) of patients weaned off treatment by the end of year 2 remained off treatment at the end of the third year (Supplemental Table 2). Of the 102 eyes followed in this group, 43% (44/102) were successfully paused (30 weeks or greater) during the course of their follow up (Supplemental Table 3; median length of follow up 28.5 months, range 12 to 72 months).

**Patients requiring maintenance therapy vs. patients weaned from anti-VEGF therapy.**

A comparison of eyes which required “maintenance treatment” every q8-12 weeks (n=35 eyes) with those who were successfully weaned off treatment by year 1 (n=32 eyes) demonstrated that the mean number of treatments received was higher in the maintenance group.
compared to the weaned group (7.8 ±0.3 vs. 6.0 ±0.3; p <0.0001; Supplemental Table 4), as expected. The mean change in CST was similar for these two groups (-80.6 ±18.6 vs. -70.4 ±13.3; p = 0.861). However, the percent of these patients with a 5-or-more-letter improvement was lower in the maintenance group compared to the weaned group (34.3% vs. 53.1%; p= 0.007; Supplemental Table 4). The percent of these patients with a 5-or-more letter decrease in vision was similar (17.1% vs. 18.8%; p = 0.713). When we specifically examined the subset of 8 eyes which were weaned from treatment but subsequently had recurrent disease activity, we observed a mean vision loss of 6 ±2 letters at the time of their recurrence (Supplemental Table 5). Following re-initiation of treatment with anti-VEGF therapy, these patients recovered 3 ±2 letters after a single re-treatment. This suggested that weaned patients who experience a recurrence of CNV remain sensitive to anti-VEGF therapy and recover vision upon resuming treatment (Supplemental Table 5).

Overall, adverse outcomes (endophthalmitis, vitreous hemorrhage, retinal tear, retinal detachment, RPE tear, subretinal hemorrhage) were uncommon. There was no increase in adverse outcomes in nvAMD patients receiving anti-VEGF therapy every 4-6 weeks compared to those receiving treatment every 8-12 weeks at 24 or 36 months (Supplemental Table 6). There was no increase in adverse outcomes in patients successfully weaned off treatment compared to patients requiring maintenance treatment (q4-6 weeks or q8-12 weeks; Supplemental Table 6).

**Confirmatory cohort of nvAMD patients treated with TEP/M**

Collectively, these results suggest that the TEP/M approach can safely and effectively wean up to 30% of nvAMD patients off anti-VEGF therapy in 12 months. To determine if the results observed in this retrospective analysis of nvAMD patients can be extrapolated to other Cao et al., 8
patient populations, we examined a second, independent cohort of patients treated using the same TEP/M protocol. To this end, we reviewed charts of a separate cohort of patients from a tertiary hospital-based clinic between 2013 to 2020 and identified 32 eyes from insured patients with a diagnosis of nvAMD (using same criteria as primary cohort by clinical exam and SD-OCT and confirmed by fluorescein angiography) who underwent intravitreal injections with anti-VEGF therapy (Supplemental Figure 3 and Table 5). Inclusions/exclusion criteria were identical to that used for the primary cohort. Patients included in this second independent cohort also agreed to participate in the TEP/M approach and were followed after initiating treatment without interruption for at least 1 year. In this second, independent cohort, 30% (6/32) of eyes were successfully weaned from anti-VEGF therapy (i.e., patients not requiring treatment on 3 consecutive scheduled visits, and for at least 30 weeks from their last injection) by the end of year 1 (Table 5), similar to what was observed in the initial cohort of patients.

**Response of fluid on OCT to anti-VEGF therapy using TEP/M in “weanable” vs. “non-weanable” patients**

We next set out to examine whether we could distinguish between patients who required frequent or maintenance therapy from those who were ultimately weaned from treatment. To this end, SD-OCT images from patients in both the initial and the confirmatory TEP/M cohorts were graded prior to initiation of treatment, at the time of diagnosis (presentation), and at 1, 2, 3, 6, and 12 months after initiating treatment for the presence of fluid. Each SD-OCT was independently classified as having no fluid (none), subretinal fluid (SRF), intraretinal fluid (IRF), or SRF and IRF (both) by two independent masked graders; disagreements were reconciled by a third grader. Patients were grouped based on frequency of treatment at 12 months Cao et al., 9
to compare those who were able to successfully be weaned from treatment with those who were unable to be weaned from treatment (Figure 1). Interestingly, while the distribution of fluid was similar in both groups at presentation, 63% (24/38) of “weanable” patients had complete resolution of fluid after their first treatment compared to only 30% (25/84) of “non-weanable” patients (Table 1). The percent of “non-weanable” patients without fluid peaked after two treatments at under 50%. Conversely, 84% (32/38) of “weanable” patients had no fluid at month two and 95% (36/38) had no fluid by month 6 (Table 1).

**Aqueous VEGF levels fail to predict response of nvAMD to anti-VEGF therapy**

These results suggest that the early response to treatment by anti-VEGF therapy may predict whether patients could ultimately be weaned from treatment. In this regard, emerging therapies under development are being designed to reduce VEGF levels more effectively (16, 17). We therefore sought to determine whether aqueous VEGF levels predict the response of nvAMD patients to treatment with current anti-VEGF therapies. A subset of patients included in our study consented to provide aqueous samples on presentation, prior to initiation of treatment with anti-VEGF therapy (Supplemental Table 7). The concentration of VEGF in these aqueous samples was measured by ELISA in patients who required monthly (q4 week), q6-8 week, or q10-12 week intervals, or patients who were weaned from treatment at the end of year 1. Compared to control patients, we observed a marked increase in the pre-treatment aqueous VEGF levels in all four groups of nvAMD patients (Figure 2A). However, we did not observe a difference in VEGF levels among these four groups (Figure 2A). Since we observed a significant difference in intraretinal and subretinal fluid on SD-OCT in weanable compared to non-weanable patients as early as 1 month after their first treatment, we next examined whether the VEGF Cao et al., 10
levels were different in patients after initiating treatment with anti-VEGF therapy. The post-treatment aqueous levels of VEGF within their first three months of initiating anti-VEGF therapy was similar to control levels and did not differ among the four subgroups of nvAMD patients (Figure 2B). Similarly, the decline in aqueous VEGF levels following initiation with anti-VEGF therapy also did not correlate with the response to treatment with anti-VEGF therapy (Figure 2C). Collectively, these results suggest that aqueous VEGF levels do not predict the response of nvAMD patients to treatment with anti-VEGF therapy.

**Proteomic analysis to identify aqueous-associated proteins from nvAMD patients.**

We next sought whether other aqueous-associated protein(s) could be identified to help predict – or may contribute to – the response to treatment with anti-VEGF therapy (Figure 3A-C). To this end, to we took an unbiased approach and examined aqueous fluid from TEP/M patients using proteomics (Figure 3D-G). Due to the limited volume of aqueous fluid available from TEP/M patients, and to reduce the influence of outliers, we performed proteomic analyses on pooled aqueous samples from untreated nvAMD patients who required monthly treatment (q4 untx group = “non-weanable”; n=3) with those nvAMD patients who required treatment every 12 weeks or could be weaned from treatment (q12+ untx group = “weanable”; n=7) at the end of year 1.

The OCT analysis of fluid in “weanable” vs. “non-weanable” TEP/M patients demonstrated no difference in the presence or absence of fluid prior to the initiation of treatment, but a measurable difference was detectable as early as 1 month after a single treatment with anti-VEGF therapy. This suggested that the difference in behavior between these two groups can be detected early after treatment initiation. We therefore also performed a proteomic analysis from

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pooled samples from treated patients (following their mandatory monthly first, second or third treatment) who required monthly treatment (q4 tx group; n=6) compared with those who required treatment every 12 weeks or could be weaned from treatment (q12+ tx group; n=5) at the end of year 1.

We detected 750 proteins in the aqueous fluid from the four pooled samples (Figure 3H). A heat map was generated comparing proteins expressed in these four groups, as well as in control patients and patients with dry (nnv)AMD (Figure 3I; Supplemental Figure 4A). Principal component analysis demonstrated a closer relationship between proteins levels in controls and nnvAMD patients compared to the two untreated groups of nvAMD patients (Figure 3J). Following treatment, the q12+ tx group was more similar to control and nnvAMD groups than was the q4 tx group (Figure 3J; Supplemental Figure 4B-D; Supplemental Table 8).

**Protein families increased or decreased in aqueous fluid from “weanable” vs. “non-weanable” nvAMD patients.**

To determine which among the 750 aqueous proteins could be used as aqueous-associated proteins that may predict the response of nvAMD patients to anti-VEGF therapy, we compared the expression levels of these proteins in the two pairs of patients. Comparison of these pairs identified 261 proteins that were increased or decreased 2-fold or more in either untx or tx q12+ groups compared to the untx or tx q4 groups (Step 1 in Figure 4A; Supplemental Figure 5). 58 proteins (Supplemental Table 9) had sequences that overlapped with the sequence of currently available anti-VEGF therapies (i.e., aflibercept, bevacizumab, or ranibizumab); this left 203 proteins that were increased or decreased 2-fold or more in the q12+ compared to the q4 groups (Step 2 in Figure 4A).

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To exclude proteins that were increased or decreased due to chance (i.e., due to the variable expression levels of the protein in the aqueous of nvAMD patients, regardless of their response to anti-VEGF therapy), we performed a proteomics analysis on a separate cohort of untreated nvAMD patients that were arbitrarily divided into two groups (nvAMD1, n=9; and nvAMD2, n=9). When we compared the proteins between these two groups, we observed 31 proteins (Supplemental Table 10; Supplemental Figure 6C) that were increased or decreased 2-fold or greater in nvAMD1 compared to nvAMD2 and that were also identified in the comparison between q4 and q12+ groups. These 31 proteins were therefore designated “highly variable” proteins and removed from the q4 vs. q12+ analyses, leaving 172 proteins that were increased or decreased 2-fold or greater in the comparison between q4 vs. q12+ aqueous samples (Step 3 in Figure 4A). Gene ontology analyses of q4 vs. q12+ untx (Figure 4B) and tx (Figure 4C) demonstrated that proteins which were increased or decreased 2-fold or more (Supplemental Tables 11 and 12) fell into several categories, including aging, angiogenesis, blood coagulation, immune response, and the response to wound healing, hypoxia, and oxidative stress, all previously implicated in the pathogenesis of nvAMD.

**Identification of proteins that may contribute to the response of nvAMD eyes to treatment with anti-VEGF therapy**

To further narrow down the proteins identified in the proteomics screen to those proteins that may serve as biomarkers or contribute to the development or progression of nvAMD, we next examined which proteins were differentially expressed in untreated nvAMD patients (n=18) compared to control patients (n=24). We observed 263 proteins that were increased or decreased 2-fold or greater in untreated nvAMD patients compared to control patients (Supplemental Cao et al., 13
To examine whether these proteins were differentially expressed in nvAMD eyes compared to control eyes (rather than variably expressed in control eyes), we arbitrarily divided a group of control patients into two groups (control 1, n=12; and control 2, n=12) and compared the expression of aqueous proteins using proteomics. Using this approach, we noted that almost half (over 47%) of proteins detected in the arbitrarily divided control groups were increased or decreased 2-fold or greater between these two groups (Supplemental Figure 6B). This was significantly greater than the diversity of protein expression observed in nvAMD patients (in which 11% of proteins were increased or decreased 2-fold or greater between the two groups; Supplemental Figure 6C). This suggested that proteomic analyses of aqueous fluid comparing nvAMD patients – and, by extension, any other disease group – with a control group can be influenced by the significant variability of protein levels among “controls” and may therefore not be helpful in identifying proteins specifically increased in patients with nvAMD.

We instead compared the expression of proteins in nvAMD patients with patients with dry (nnv)AMD (n=18) and identified 109 proteins that were differentially expressed (by at least 2-fold) in nnvAMD vs. nvAMD and may therefore contribute to the progression of dry to wet AMD (Supplemental Figure 6D). Gene ontology analyses revealed the same categories identified in the comparison between q4 and q12+ groups (Figure 5A). Indeed, among the 109 proteins identified in the nvAMD vs. nnvAMD comparison, 42 overlapped with proteins identified in the q12+ untx group compared to the q4 untx group, 18 overlapped with proteins identified in the q12+ tx group compared to the q4 tx group, and 8 proteins were identified in all three comparisons (Figure 5B; Supplemental Tables 13 and 14).
Complement proteins differentially expressed in aqueous fluid from “weanable” vs. “non-weanable” nvAMD patients.

Recent data strongly implicate immune dysregulation in the development of AMD (18). Genome-wide association studies have identified several variants of the innate immune system complement genes that influence the risk of developing AMD (19). Among the proteins that were increased or decreased 2-fold or more in q4 vs. q12+ groups were several immunomodulatory proteins (Figure 6A). This included 6 complement-related proteins: 4 that were increased or decreased 2-fold or greater in the q12+ untx group compared to the q4 untx group and 2 that were increased or decreased 2-fold or greater in the q12+ tx group compared to the q4 tx group (Supplemental Table 15). One of these complement-related proteins was identified as “highly variable” (Supplemental Table 10), leaving 5 complement-related proteins: C3, complement factor 4-A (C4-A), complement Factor H-related (CFHR) protein 2, CFHR 4, and CFHR 5 (Figure 6B; Supplemental Table 15). In the comparison of nnvAMD vs. nvAMD, we identified 3 additional complement-related proteins that were increased or decreased 2-fold or greater: complement factor 1, complement component C8 beta chain, and CFHR 4.

Proteins that respond differently in aqueous fluid from “weanable” vs. “non-weanable” nvAMD patients.

As stated above, the OCT analysis of fluid in “weanable” vs. “non-weanable” TEP/M patients demonstrated no difference in the presence or absence of fluid prior to the initiation of treatment, but a measurable difference was detectable as early as 1 month after a single treatment with anti-VEGF therapy. We therefore set out to identify proteins that respond differently in q4 vs. q12+ patients following initiation with treatment of anti-VEGF therapy. To this end, we

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compared the expression of proteins identified in the proteomic analyses in the q4 untx group with the q4 tx group and the q12+ untx group with the q12+ tx group (Figure 7A-D). Interestingly, while 16 of the 42 proteins identified in all three proteomics screens (q4 vs. q12+ untreated; q4 vs. q12+ treated; and nvAMD vs. nnvAMD) behaved similarly in both the q4 and q12+ groups (Figure 7A and B), 9 proteins increased in the q4 group but decreased in the q12+ group in response to treatment (Figure 7C) while 17 proteins decreased in the q4 group but increased in the q12+ group in response to treatment (Figure 7D), suggesting that they may directly contribute to the different response of q4 vs. q12+ nvAMD patients to anti-VEGF therapy. Of these 26 proteins, 4 were among the 8 proteins differentially expressed (2-fold or more) in all three comparisons (q4 vs. q12+ untreated; q4 vs. q12+ treated; and nvAMD vs. nnvAMD; see Figure 5B).

**Apolipoprotein B-100 plays a protective role in the development of CNV.**

Among the 4 proteins that responded differently in the q4 compared to q12+ groups following treatment, and that were present in all three comparisons (q4 vs. q12+ untreated; q4 vs. q12+ treated, and nvAMD vs. nnvAMD) was apolipoprotein B100 (ApoB100). ApoB100 expression decreased in the q4 group but increased in the q12+ group following treatment (Figure 7D). ApoB100 has been reported to be secreted by the retinal pigment epithelium (20-24), and accumulates within Bruch’s membrane as an early component of drusen (25-29). It is speculated that oxidized ApoB100 may contribute to the development of (dry) nnvAMD and the progression to (wet) nvAMD (30). However, our results demonstrate a paradoxical increase in ApoB100 in the eyes of patients who respond better to anti-VEGF therapy. To corroborate the results from our proteomics studies, we measured by ELISA the aqueous ApoB100 levels in an
unrelated cohort of patients with nvAMD, untreated nvAMD, as well as non-AMD control patients (Supplemental Table 16). We observed an increase in aqueous ApoB100 levels in patients with early and intermediate nvAMD compared to control patients (Figure 8A), consistent with a role for ApoB100 in the development of dry AMD. The aqueous ApoB100 levels in nvAMD patients was similarly increased compared to controls. However, aqueous ApoB100 levels were significantly higher in patients with early and intermediate nvAMD patients compared to patients with nvAMD (Figure 8A), suggesting that ApoB100 levels decrease with progression from dry to wet AMD.

Based on the observation that aqueous ApoB100 levels were increased in treated nvAMD patients who required infrequent treatment compared to those who required monthly treatment, as well as the reduced aqueous ApoB100 levels in nvAMD patients compared to those with early and intermediate nvAMD, we hypothesized that expression of ApoB100 may play a protective role in nvAMD. To interrogate this hypothesis, we took advantage of a homozygous mutant mouse line in which a CTA to TTA mutation was introduced to sequences corresponding to the ApoB48 editing codon (codon 2179) in exon 26, resulting in a marked reduction of ApoB48 expression (31). Expression of ApoB in these mice therefore is shifted from 50% ApoB100 and 50% ApoB48 to over 90% ApoB100. qPCR analysis demonstrated a marked increase in the mRNA expression of the *ApoB100* isoform in the RPE/choroid from ApoB mutant mice compared to wild type mice (Figure 8B). Aged (9-month-old) ApoB mutant animals were subjected to laser treatment and the size of CNV lesions was measured 7 days later. We observed smaller CNV lesions in the *ApoB* mutant mice compared to wild type mice (Figure 8C), consistent with a protective role for ApoB100 in nvAMD.

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Although their development is normal, ApoB mutant mice have lower HDL cholesterol levels than wildtype mice. Over time this, in turn, leads to lipoprotein accumulation in Bruch’s membrane (20). To examine whether the effects on CNV lesion size in ApoB mutant mice was due to expression of ApoB100 in the RPE/choroid rather than to lipid deposition and alterations of Bruch’s membrane, we examined the development of CNV lesions in younger animals and observed a similar reduction in CNV lesion size in 3-month-old ApoB mutant mice compared to control wild type mice (Figure 8D).

We next examined whether increased expression of ApoB100 influenced VEGF expression or the response of CNV lesions to anti-VEGF therapy. To this end, we first examined the expression of Vegf mRNA by qPCR in the RPE/choroid from ApoB mutant mice compared to wild type mice. We observed similar levels of Vegf mRNA in both ApoB mutant and wild type mice (Figure 8E). We next treated ApoB mutant mice with a single intraocular injection with aflibercept (200 ng) at day 3 following laser treatment. Examination of animals at day 7 demonstrated further reduction in the size of the CNV lesion in ApoB mutant mice, similar to wild type control mice (Figure 8F). CNV lesions were smaller in ApoB mutant mice treated with aflibercept compared to wild type mice treated with aflibercept (Figure 8F). Collectively, these data suggest that ApoB100, an early and key component of drusen, could play an unexpected protective role in the development of CNV in patients with nvAMD, and that this role may be independent of Vegf mRNA expression and additive to anti-VEGF therapy.

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**Discussion**

It is anticipated that the number of people who have AMD will approach 300 million by 2040 (32). Close to 14 million people in the United States have AMD (33), a number that increases by almost 300,000 every year (34). Despite affecting only 10% of patients with AMD, CNV is the cause of severe vision loss in 90% of AMD patients (35), and remains the leading cause of blindness among the elderly in the United States (36). The introduction of anti-VEGF therapies has had a remarkable impact on nvAMD patients who previously suffered major, irreversible vision loss from edema, bleeding or scarring caused by CNV (2). Although calculating the economic impact of preventing vision loss – or improving vision – in treated patients is challenging, there is little doubt that anti-VEGF therapy has improved the quality of life of millions of patients worldwide. Current efforts are now focused on efforts to refine treatment protocols to reduce the number of injections (and treatment-related adverse events) without sacrificing the visual acuity benefits observed with monthly or bi-monthly treatment.

Prior clinical trials have demonstrated that the TAE approach was not inferior to monthly treatment with anti-VEGF therapy, but reduced the number of treatments by 25% in 2 years (15). Several subsequent studies have shown similar success using TAE, with the benefit of reducing patient visits and injection burden, while preserving visual outcomes (14, 37-40). It has also been reported that switching from PRN to TAE results in an improvement in both CST and visual acuity (41, 42). However, the requirement for maintenance therapy in nvAMD patients remains under debate.

Here, we use a hybrid of the TAE and PRN approaches, treat-and-extend-pause/monitor (TEP/M), to optimize the efficacy of anti-VEGF therapy for each specific patient, while minimizing the number of treatments needed. Compared to traditional bi-monthly treatment

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(following 3 initial monthly treatments), TEP/M resulted in a 27% decrease in the mean number of treatments after two years (10.2 ±0.9 vs. 14). Compared to traditional monthly treatment, the number of treatments using the TEP/M protocol was reduced by 44% (25 vs. 14.0 ±0.8) at two years. These results demonstrate that TEP/M effectively reduces the number of treatments and extends the interval between visits for anti-VEGF therapy. Using the TEP/M protocol, we further observed that (32/102) 31% of nvAMD patients could be weaned off treatment within 1 year. Re-treatment of patients was required in only (6/22) 27% of patients in the second year following initiation of the treatment pause. By the end of year two, (25/65) 38% of nvAMD patients had been weaned off treatment, with only (2/15) 13% of patients requiring re-treatment in their third year. This is a dramatic departure from current treatment strategies which require indefinite treatment for nvAMD. We corroborate these findings in a second independent cohort of patients and observed similar results.

Two prior reports have previously evaluated a hybrid TAE/PRN approach to wean nvAMD patients off treatment (43, 44). In the first study, patients receiving anti-VEGF therapy underwent a TAE protocol once the macula was determined to be dry on SD-OCT (43), similar to our study. Although this prior report did not utilize a strict protocol for treatment extension, it was reported that the interval between visits was extended by 1 or 2 weeks if there was no fluid on SD-OCT. Once patients were extended to 12 weeks, they received treatment, followed by 2 additional maintenance treatments 12 weeks apart (for almost 9 months) before they were taken off treatment (after a minimum of 8 injections over approximately 16 months). Using this approach, 143 out of 385 eyes (37.3%) were successfully weaned off treatment; this is similar to what we observed here after 1 year. However, in the prior study, the time interval required to successfully wean these patients off treatment was not reported. Additionally, the authors Cao et al., 20
included in the “successfully weaned off treatment” group any patient who was taken off treatment at any time during the study, over the entire follow-up period, and no criteria were set to exclude patients who were non-adherent with their regimen or follow-up visits.

A more recent study also examined the potential of weaning nvAMD patients off treatment with anti-VEGF therapy using a TAE protocol (44). While the investigators began extending the interval between visits after the second treatment, the interval plateaued at 16 rather than 12 weeks, and was followed by 3 additional maintenance treatments 16 weeks apart (i.e., for almost 1 year) before holding treatment and entering a monitoring phase during which patients were examined every 3-4 months. 17% (100 out of 598 eyes) of eyes reached exit criteria. These patients required a minimum of 10 injections over 2 years with an average treatment duration of 4.5 years (range of 2-7 years).

These studies included broad inclusion criteria for patient selection, with unclear adjustments for prior treatment, comorbid ischemic retinopathies, or other underlying disease. While both studies utilized SD-OCT at all initial and follow-up visits, FA was not required for confirmation of diagnosis in one study (44). Importantly, patients who did not adhere to regular treatment intervals were still included in their analyses (43, 44).

Limitations of the clinical portion of our study include that it is a retrospective study with a limited number of patients, the follow up is 1 year (with a subset of patients extended out for 2 or 3 years), and best corrected visual acuity (BCVA) was not obtained at each visit. Nonetheless, in our study, diagnosis of nvAMD by clinical exam and SD-OCT was confirmed by FA. And patients with a second diagnosis that could influence their response to treatment were excluded from the study. Moreover, our results were corroborated in a second independent cohort of patients. We also required strict adherence to the TAE protocol; significant (defined) deviations

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from the protocol led to exclusion of the patient from our analysis. We did not include any maintenance therapy period, allowing us to rapidly wean patients off treatment within 8 months of treatment initiation and with as few as 6 injections compared to 8-10 injections over 16-24 months (43, 44). Despite this rapid weaning approach with our TEP/M protocol, 31% of all patients were successfully weaned off treatment by 1 year. This rapid approach is supported by our OCT analysis which demonstrates a clear distinction in the response to treatment between “non-weanable” and “weanable” patients as early as 1 month after treatment. Interestingly, the CST was similar in patients weaned from treatment compared to patients who required maintenance doses (q8-12 weeks). Moreover, more patients weaned from treatment experienced a 5-or-more letter improvement in vision compared to patients requiring maintenance therapy. There was no difference in adverse outcomes in patients weaned off treatment compared to patients receiving q4-6 weeks or q8-12 week treatment. And patients who were weaned from treatment and subsequently had a recurrence of CNV remained sensitive to resuming treatment with anti-VEGF therapy. Collectively, these results demonstrate the safety and efficacy of the TEP/M approach.

These studies have important implications for management of patients with nvAMD. The potential long-term cost-savings for nvAMD patients successfully weaned of treatment will have an important impact on cost-effective analyses of different anti-VEGF therapies. Moreover, our results suggest that up to one third of nvAMD patients may not require – or benefit from – the anticipated introduction of second-generation, longer-acting anti-VEGF therapies (Supplemental Figure 7) (45). This is particularly important given emerging concerns of the untoward consequences of long-term suppression of VEGF in the eyes of AMD patients (10).

Our results further suggest that patients with nvAMD are not a homogeneous population;

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but instead are comprised of three distinct subgroups (Supplemental Figure 7): patients who respond inadequately despite monthly anti-VEGF therapy (q4 patients; approximately 20%); patients who respond anti-VEGF therapy, but require indefinite treatment (approximately 50%); and patients who can be weaned off treatment (q12+ patients; approximately 30%). These subgroups can be distinguished by their early response to treatment, as reflected by their SD-OCT images. Moreover, we demonstrate that pre- and post-treatment aqueous VEGF levels could not predict which patients will require monthly treatment compared to those patients who could be extended to 6-12 weeks or weaned from treatment. These results suggest that additional factors may influence how nvAMD patients respond to anti-VEGF therapy.

These findings also have important clinical implications for patients with nvAMD. They suggest that there are definable subgroups of nvAMD patients who respond differently to anti-VEGF therapy. It is also not known whether specific anti-VEGF therapies may be more effective than others at weaning patients off therapy. The ability to wean patients off therapy may have a significant impact on the long-term cost-effectiveness of anti-VEGF therapies. These unanswered questions are under current investigation. This is particularly relevant given emerging ethical issues about the distribution of “cost-effective” vs. “most effective” anti-VEGF therapies among patients with ocular neovascular disease (46).

The ability to determine how patients will respond to anti-VEGF therapy will also be essential for deciding which patients will benefit from the next generation of longer-acting anti-VEGF therapies vs. those who may be successfully (and transiently) treated with currently available therapies. In the second half of this study, we therefore took an unbiased approach to identify aqueous biomarkers that may help distinguish between (or contribute to) these subgroups of nvAMD patients. Using proteomics, we identified 172 proteins which were

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differentially expressed in the aqueous of untreated or treated q12+ compared to q4 patients. Gene ontology analyses demonstrated that these proteins fell into several families, including aging, angiogenesis, blood coagulation, the immune response, and the response to wound healing, hypoxia, and oxidative stress, all previously connected with AMD pathogenesis. These results suggest that aqueous levels of a subgroup of specific proteins could serve as biomarkers which may help predict which patient would benefit from new longer-acting therapies or therapies targeting additional vasoactive mediators. Future studies will be needed to identify which of these proteins that could comprise an array of biomarkers which would help predict how nvAMD patients will respond to current and future therapies. Of note, our proteomic analysis of non-AMD control patients demonstrated remarkable variability within this group; this suggests that caution should be taken when using aqueous fluid from “control” patients in proteomic analyses.

Many of the proteins identified in the proteomic analyses have previously been implicated in the pathogenesis of AMD, suggesting that they may further play a functional role in determining which patients respond adequately to anti-VEGF therapy. In this regard, among the proteins we identified that were differentially expressed in our proteomic analyses were 8 complement-related proteins. The complement system plays an important role in the pathogenesis of AMD (19). Drusen, present in all patients with AMD, are comprised of proteins involved in inflammation and include components of the complement system (30). There is strong genetic evidence for an association of variants in the genes of the complement system, including C3 and CFH and the development of advanced AMD (19). While patients with AMD exhibit elevated serum levels of activation products complement proteins, the role of these complement proteins in the development of AMD remains unclear. One of the 8 complement-
related proteins identified in our proteomics studies was C3. C3 was 2-fold higher in patients requiring monthly treatment with anti-VEGF therapy compared to those requiring less frequent treatment. This observation is consistent with prior studies suggesting that C3 activation may contribute to AMD pathogenesis.

Of the remaining 7 complement-related proteins identified in our proteomics studies, 3 were members of the CFHR family of proteins. Variants of the complement inhibitor CFH have also been reported to increase the risk for developing AMD (47). CFHR proteins are evolutionarily and structurally related to CFH and have previously been implicated in other diseases involving complement dysregulation (48). However, the CFHR proteins do not appear to share CFH’s role as a negative complement regulator. Rather, recent evidence suggests that CFHR proteins may instead enhance complement activation. CFHR4 was more than 6-fold lower in patients requiring monthly treatment with anti-VEGF therapy compared to those requiring less frequent treatment. Conversely, CFHR2 and CFHR5 were 2-fold higher in patients requiring monthly treatment with anti-VEGF therapy compared to those requiring less frequent treatment. Whether CFHR proteins contribute to the development of nvAMD, or the response of nvAMD patients to anti-VEGF therapy, requires further investigation.

We further identify eight proteins differentially expressed (2-fold or more) in all three comparisons (q4 vs. q12+ untreated; q4 vs. q12+ treated; and nvAMD vs. nnvAMD). These proteins included hemoglobin subunits α and β, carbonic anhydrase 1, fructose-bisphosphate aldolase A, keratin, type II cytoskeletal 5, ApoB100, HSP 90β, and semaphorin-4B. Among these 8 proteins, the latter 4 proteins responded differently in the q4 compared to q12+ groups following treatment with anti-VEGF therapy, suggesting that they may influence the response of nvAMD patients to treatment. Among these proteins, ApoB100 is of particular interest in AMD.
pathogenesis. ApoB100 has been reported to be secreted by the retinal pigment epithelium (20-24), and accumulates within Bruch’s membrane as an early component of drusen (25-29). It is speculated that oxidized ApoB100 may contribute to the development of (dry) nnvAMD and the progression to (wet) nvAMD (30). However, our results suggest ApoB100 may also play a novel and paradoxical protective role in (wet) nvAMD. We observed that aqueous levels of ApoB100 were higher in patients with early and intermediate nnvAMD compared to control patients, but lower in nvAMD patients compared to nnvAMD patients. These observations suggest that increased ApoB100 expression correlates with the development of nnvAMD, but lower ApoB100 levels correlates with nvAMD. Indeed, we observed that mutant mice over-expressing ApoB100 in the RPE/choroid developed smaller CNV lesions in the laser-induced CNV model compared to wild type controls. Moreover, the influence of ApoB100 on CNV lesions was independent of Vegf mRNA expression and additive to anti-VEGF therapy.

Collectively, these observations implicate ApoB100, a key component of drusen, in the protection against the development of nvAMD. In light of these unexpected findings, it may be prudent to re-visit the role of drusen – or components of drusen – in the pathogenesis of AMD. In this regard, we also observed differential expression of apolipoprotein A and apolipoprotein D in treated nvAMD patients who required monthly treatment with anti-VEGF therapy compared to those requiring less frequent treatment. Future studies may help determine whether (and how) these lipoproteins – as well as the other proteins identified in these proteomics studies – contribute to the development of nnvAMD vs. nvAMD, or to the response of nvAMD patients to anti-VEGF therapy.

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Methods

Clinical Studies

Please see Supplemental Information for details of clinical studies.

Aqueous Samples

Institutional Review Board approval from the Johns Hopkins University School of Medicine (Baltimore, MD) was obtained for all patient samples used in this HIPAA-compliant study. Aqueous samples (0.1 – 0.2 mL) were collected via limbal paracentesis using a 30-gauge needle attached to a tuberculin syringe from consenting patients at the Wilmer Eye Institute immediately after performing intravitreal injection for active CNV. Consent was written and voluntary without stipend. Aqueous samples were immediately processed and stored at -80°C prior to analysis.

ELISA

Human VEGF (Duoset) and ApoB100 (Quantikine) ELISA kits were purchased from R&D Systems (Minneapolis, MN). Aqueous samples were analyzed for VEGF and ApoB100 (10 μL of aqueous diluted 1:10) using ELISAs, and performed according to the manufacturer’s protocols. All ELISAs were performed in duplicate (VEGF) or triplicate (ApoB100) and quantitation was performed using the standard curve constructed with the standards included in the kit.

Proteomics

Please see Supplemental Information for details of proteomics analyses.

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**Animal Studies**

Please see Supplemental Information for details of animal studies.

**Statistical Analysis**

Categorical variables were presented as percentages and compared using the two-sided chi-squared test with significance set at P<0.05. Patient’s BCVAs were converted from Snellen Visual Acuity into logarithm of the minimum angle of resolution (logMAR) score for statistical analysis. LogMar was converted into approximate Early Treatment Diabetic Retinopathy Study (ETDRS) scores to determine mean gains or losses of letters at 12 and 24 months compared to their initial vision prior to treatment initiation. Data for continuous variables were recorded as mean ± standard error of the mean (SEM). Assuming nonparametric data, an unpaired, 2-tailed, Mann-Whitney Test analysis with significance set at P<0.05 was used to compare mean data points in this study. Scatterplots for VEGF and ApoB100 were generated using MATLAB. For the animal studies, statistical differences between two heterogenous groups were determined by Unpaired Student’s t test and for more than two groups were determined by one-way ANOVA with Turkey’s multiple comparison test. Statistical analyses were performed using Microsoft Excel and GraphPad Prism 8.0 software (GraphPad, San Diego, California). One-way ANOVA was used to perform statistical testing for age difference of patients in the proteomics analysis, VEGF ELISA, and ApoB100 ELISA assays. The Wilxocon Rank Sum test was used for statistical analysis between patient groups in pre- and post-treatment VEGF ELISAs. Adjusted p-values were calculated using the FDR method to account for multiple comparisons. Fisher’s exact test was used to perform statistical testing for sex and pseudophakic difference. Both One-Way ANOVA and Fisher’s exact test were carried out in R environment (4.0.5).

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Statistical significance was defined as $P$ less than 0.05. $*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$; NS, nonsignificant.

**Study Approval**

Institutional Review Board approval from the Johns Hopkins University School of Medicine (Baltimore, MD) was obtained for all patient information, including OCT images and aqueous samples, used in this study. All experiments involving animals were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and formally reviewed and approved by the Johns Hopkins University Animal Care and Use Committee Animal Research Reporting.
Acknowledgment

This work was supported by National Eye Institute, National Institutes of Health grants R01EY029750 to A.S. and J.Q., R01EY025705 to S.M. and A.S., and R01 EY27961 to J.T.H. A.S. gratefully acknowledges the support he received as a Special Scholar Award recipient from Research to Prevent Blindness, Inc., as recipient of the Alcon Young Investigator Award from the Alcon Research Institute, and from the Branna and Irving Sisenwein Professorship in Ophthalmology. J.T.H is supported by the Robert Bond Welch Professorship. A.S. is also supported by an unrestricted grant from Research to Prevent Blindness, Inc.. The authors thank Dr. Marisol Cano for her help with the animal studies.

Akrit Sodhi, M.D., Ph.D., had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
References


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Figure 1. Heatmap comparing fluid over time for eyes of patients who required sustained anti-VEGF treatment vs. those who were successfully weaned from anti-VEGF therapy by 12 months. OCT images were obtained from all 102 eligible patients who underwent the TEP/M approach for at least 12 months. Presence of fluid on OCT was graded independently by two investigators (TPP, ZY) for the presence of no fluid, subretinal fluid (SRF), intraretinal fluid (IRF), or both at timepoints 0, 1, 2, 3, 6, and 12 months after initiation of protocol. Fluid status overtime for each individual patient is graphically represented with dark blue denoting no fluid, light blue (SRF), light green (IRF), and yellow (both). Patients were grouped into 2 categories – those not weaned (requiring sustained treatment every 4 – 12 weeks), and those weaned off treatment. Within each group, patients were sorted by severity of fluid (none < SRF < IRF < both).
Figure 2

A) Pre-treatment Aqueous [VEGF] (pg/mL) weaned (n=9) q10-12 weeks (n=3) q6-8 weeks (n=5) q4 weeks (n=6) control (n=23)

B) Pre-treatment Aqueous [VEGF] (pg/mL) weaned (n=3) q10-12 weeks (n=4) q6-8 weeks (n=1) q4 weeks (n=4) control (n=23)

C) Pre-treatment Aqueous [VEGF] (pg/mL) q4 weeks (n=4) q6-8 weeks (n=1) q10-12 weeks (n=4) weaned (n=3)
Figure 2. Aqueous levels of HIF-regulated vasoactive mediators in TEP/M patients. (A) Pre-treatment aqueous VEGF levels (prior to first injection) for patients with increasing interval between treatments at 12 months (from subset of TEP/M patients). (B) Post-treatment aqueous VEGF levels (having received their first injection) for patients with increasing interval between treatments at 12 months (from subset of TEP/M patients). (C) Comparison of Pre-treatment and Post-treatment aqueous VEGF levels for patients with increasing interval between treatments at 12 months (from subset of TEP/M patients). (Statistical analysis was performed using Wilcoxon Rank Sum test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, non-significant. x = statistical analyses could not be performed due to insufficient samples.)
Figure 3

Aqueous Samples Obtained from nvAMD Patients

q4 nvAMD patients

q12+ nvAMD patients

Active CNV

VEGF & Other?

10 µg Protein/Sample

Lys-C and Trypsin Digestion Into Peptides

Data-Independent Acquisition Mass Spectrometry (DIA-MS)

Differential Protein Analysis Comparing:

Controls

nnvAMD

q4 nvAMD

q12+ nvAMD

Untreated

Treated

Non-AMD Control

nvAMD

750 Aqueous Proteins

Protein Identification (Spectronaut)

3D Principal Component Analysis (PCA)
Figure 3. Overview of patient sample collection and proteomics screen in untreated (anti-VEGF naïve) and treated (those who have received anti-VEGF therapy) wet AMD patients. (A) Aqueous samples were collected from clinic patients with neovascular Age-Related Macular Degeneration via anterior chamber paracentesis. (B and C) Illustration of disrupted outer blood-retinal barrier in neovascular Age-Related Macular Degeneration (B) and release of VEGF and other angiogenic mediators that promote choroidal neovascularization (C). (D) Patient samples were separated into those which required monthly (Q4) treatment vs. those who were able to be extended to 12 or more weeks (Q12+) and by anti-VEGF treatment status (treatment Naïve and post treatment) (E) Pooled patient aqueous samples containing 10 ug of protein were prepared (F) Samples were digested by proteolytic enzymes (G) Mass Spectrometry was performed to analyze each sample (H) Unique Proteins were identified using the Spectronaut Proteomics System (I) Differential Protein Analysis was performed comparing Q4 and Q12+ NV AMD patient samples with non-AMD control and Dry AMD patients (J) To evaluate the molecular effects of anti-VEGF treatment in patients, we utilized Principal component analysis (PCA). The samples used in the analysis include Q4 and Q12+ patients and their response to anti-VEGF treatment, as well as non-AMD controls and Dry AMD patients. The first 3 components were selected for the 3D PCA.
A 750 proteins detected by proteomics screen of aqueous fluid from nvAMD patients

Step 1

261 proteins increased or decreased 2-fold or more in untreated q12+ vs. q4 (195), treated q12+ vs. q4 (102)

Step 2

203 Proteins increased or decreased 2-fold or more

Step 3

172 Proteins increased or decreased 2-fold or more in untreated q12+ vs. q4 (130), treated q12+ vs. q4 (61) (see Supplemental Tables 11 and 12)

Removal of proteins with less than 2-fold difference in expression levels in comparison groups

Removal of 58 proteins with overlapping sequences with anti-VEGF therapies (see Supplemental Table 9)

Removal of 31 "highly variable" proteins (see Supplemental Table 10)

B

regulation of immune response
immune response
blood coagulation
blood coagulation, intrinsic pathway
innate immune response
visual perception
aging
response to hypoxia
response to oxidative stress
inflammatory response
angiogenesis
regulation of immune system process

C

regulation of immune response
immune response
blood coagulation
blood coagulation, intrinsic pathway
innate immune response
visual perception
aging
response to oxidative stress
inflammatory response
angiogenesis
Figure 4. Stepwise Identification and Isolation of key proteins driving phenotype of nvAMD patients in untreated and treated q12+ vs q4 groups. (A) Flow diagram describing process of removing proteins with similar expression levels, those with sequences that overlapped with anti-VEGF therapies, and those that were highly variable between q4 and q12+ groups to identify proteins of interest. (B) Scatter plot of identified aqueous proteins with 2-fold changes between q4 untreated patients and q12+ untreated patients. Colored markers represented enriched biological process from the proteomics analysis. Gray dots were the proteins with no enriched biological processes. (C) Scatter plot of identified aqueous proteins with 2-fold changes between q4 treated patients and q12+ treated patients. Colored markers represented enriched biological process from the proteomics analysis. Gray dots were the proteins with no enriched biological processes.
Figure 5

A

Key aqueous proteins driving phenotype of nvAMD patients

B

172 Key aqueous proteins driving phenotype of nvAMD patients

q12+ untreated
vs. q4 untreated

q12+ treated
vs. q4 treated
nvAMD vs. nnvAMD
Figure 5. Comparison of Expression of Key Proteins in nvAMD vs. nnvAMD and identification of overlapping proteins between q4 vs. q12 untreated patients, q4 vs. q12 treated patients, and nnv vs. nvAMD patients. (A) Scatter plot of identified aqueous proteins with 2-fold changes between (dry) nnvAMD patients and (wet) nvAMD patients. Colored markers represented enriched biological process from the proteomics analysis. Gray dots were the proteins with no enriched biological processes. (B) Venn Diagram describing identification of overlapping proteins that were identified in the comparisons between q4 vs. q12 untreated patients, q4 vs. q12 treated patients, and nnv vs. nvAMD patients (see supplemental table 12).
Figure 6. Comparison of Expression of Key Immunomodulatory Proteins and Complement Proteins in q4 vs. q12 untreated patients, q4 vs. q12 treated patients, and nnv vs. nvAMD patients. (A) Comparison of the expression of aqueous proteins identified in the proteomics analyses between q4 vs. q12 untreated patients, q4 vs. q12 treated patients, and nnv vs. nvAMD patients highlighting immunomodulatory proteins. Proteins increased or decreased less than 2-fold were excluded from this analysis. (B) Comparison of the expression of aqueous proteins identified in the proteomics analyses between q4 vs. q12 untreated patients, q4 vs. q12 treated patients, and nnv vs. nvAMD patients highlighting complement proteins.
Figure 7

A

B

C

D

Log[Aqueous Protein]

Log[Aqueous Protein]

Log[Aqueous Protein]

Log[Aqueous Protein]

Tx  UnTx  Tx  UnTx

q4  q12+

q4  q12+

q4  q12+

q4  q12+

Haptoglobin
Fructose-bisphosphate aldolase A
Beta-crystallin B2
Retinoid acid receptor responder protein 1
Carbonic anhydrase 1
Carbonic anhydrase 2
Mimic
C4b-binding protein beta chain
Peroxiredoxin-2
Hemoglobin subunit beta
Hemoglobin subunit alpha
Fatty acid-binding protein 5
Peroxiredoxin-1
Uncharacterized protein C18orf63
Follistatin-related protein 4
Beta-crystallin A3

Ficolin-3
Alpha-crystallin B chain
Apolipoprotein B-100
Heat shock protein beta-1
Vimatin
Heat shock protein HSP 90-beta
Keratin, type II cytoskeletal 5
Macrophage migration inhibitory factor
Pyruvate kinase PM
Bilgycan
Insulin-like growth factor-binding protein complex acid soluble subunit
Beta-crystallin A4
Actin, cytoplasmic 2
Semaphorin-3A
Clusterin-like protein 1
Cocaine- and amphetamine-regulated transcript protein
Semaphorin-4B

Serine/threonine-protein kinase/erbb-mutant-susceptible gene 1
Corticosteroid-binding globulin
Ubiquitin carboxyl-terminal hydrolase isozyme L1
Polyubiquitin-B
Serpin B6
14-3-3 protein epsilon
Pepidyl-prolyl cis-trans isomerase A
Complement factor H-related protein 4
Protein/nucleic acid deoxyribonuclease DJ-1

Bilgycan
Insulin-like growth factor-binding protein complex acid soluble subunit
Beta-crystallin A4
Actin, cytoplasmic 2
Semaphorin-3A
Clusterin-like protein 1
Cocaine- and amphetamine-regulated transcript protein
Semaphorin-4B
Figure 7. “Key” proteins identified by proteomics analyses and their response to anti-VEGF treatment in q4 vs. q12 patients. (A) Proteins that increased following treatment with anti-VEGF therapy in both q4 and q12+ nvAMD patients. (B) Proteins that decreased following treatment with anti-VEGF therapy in both q4 and q12+ nvAMD patients. (C) Proteins that increased following treatment with anti-VEGF therapy in q4 nvAMD patients but decreased following treatment in the q12+ nvAMD patients. (D) Proteins that decreased following treatment with anti-VEGF therapy in q4 nvAMD patients but increased following treatment in the q12+ nvAMD patients. Red box highlights proteins present in all three comparisons (q4 vs. q12+ untreated; q4 vs. q12+ treated, and nvAMD vs. nnvAMD; see Figure 5B).
Figure 8

A

Non-AMD Control (n=10)  NnvAMD (n=10)  NvAMD (n=10)

Area of lesions (mm²)

B

wt  ApoB mutant

Apob 100 mRNA (fold change)

C  D

E

Vegf mRNA (relative expression)

F

Area of lesions (mm²)
Figure 8. (A) ApoB100 protein level in aqueous of non-AMD control, NnvAMD (Non-neovascular AMD), and NvAMD (Neovascular AMD). Compared with that of non-AMD control, ApoB100 was significantly higher in the aqueous of NnvAMD and NvAMD patients. Moreover, ApoB100 levels in the aqueous of NnvAMD patients was dramatically higher than that in NvAMD patients. (B) ApoB100 mRNA level in neurosensory retinal, RPE/choroid, and liver of both wt and ApoB mutant mice. ApoB100 level in all three tissues of ApoB mutant are significantly higher than that from wt mice. (C and D) Laser CNV lesion size in 9-month old (C) and 3-month old (D) wt and ApoB100 mutant mice 7 days following treatment with laser. Individual dots represent CNV spots from choroids of four mice in each case. A reduction in choroidal neovascularization was observed in ApoB100 transgenic mice compared to wt mice. Results were plotted as mean ± SD. Unpaired student t test was used to compare the between the two groups with each other. *, p<0.05. (E) Vegf mRNA level in RPE/choroid from wt and ApoB mutant mice. Results were plotted as mean ± SD. P-values were generated by two-tailed student’s t-test. (F) Laser CNV comparison between wt and ApoB mutant mice 7 days following treatment with laser. A subset of mice were treated with 200 ng aflibercept on day 3 following laser treatment. n=4-8 animals for each condition. Results were plotted as mean ± SD. One-way ANOVA with Turkey’s multiple comparison test was used to compare the different treatments with each other. ****, p<0.0001; ***, p<0.001; **, p<0.01; *, p<0.05; and NS, p>0.05.
Table 1. Presenting characteristics of eyes of nvAMD patients receiving anti-VEGF therapy and those eligible for study at 12 and 24 month time points.

<table>
<thead>
<tr>
<th>Month</th>
<th>Non-weaned n = 84</th>
<th>Weaned n = 38</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2% (1)</td>
<td>0.0% (0)</td>
<td>0.497</td>
</tr>
<tr>
<td>1</td>
<td>29.8% (25)</td>
<td>63.2% (24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>46.2% (39)</td>
<td>84.2% (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>48.8% (41)</td>
<td>84.2% (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>48.8% (41)</td>
<td>94.7% (36)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: No., number; TEP/M, treat and extend pause/monitor. Values displayed as mean ± standard error of the mean. Statistical analysis was performed using Chi-square and Mann-Whitney test. Data in bold are statistically significant.
Table 2. Outcome measurements for TEP/M patients at 12 months.

<table>
<thead>
<tr>
<th>Characteristics at 12 months</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Number of Eyes</td>
<td>102</td>
</tr>
<tr>
<td>Vision (ETDRS letters)</td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>End</td>
<td>57± 3</td>
</tr>
<tr>
<td>Change</td>
<td>+2 ± 2</td>
</tr>
<tr>
<td>Letters gained - % (no.)</td>
<td></td>
</tr>
<tr>
<td>5 or more letters</td>
<td>46% (47)</td>
</tr>
<tr>
<td>10 or more letters</td>
<td>30% (31)</td>
</tr>
<tr>
<td>15 or more letters</td>
<td>16% (16)</td>
</tr>
<tr>
<td>Letters lost - % (no.)</td>
<td></td>
</tr>
<tr>
<td>5 or more letters</td>
<td>19% (19)</td>
</tr>
<tr>
<td>10 or more letters</td>
<td>14% (14)</td>
</tr>
<tr>
<td>15 or more letters</td>
<td>13% (13)</td>
</tr>
<tr>
<td>CST (µm)</td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>323.6 ± 10.0</td>
</tr>
<tr>
<td>End</td>
<td>244.3 ± 51</td>
</tr>
<tr>
<td>Change</td>
<td>-79.3 ± 9.6</td>
</tr>
<tr>
<td>Mean # of treatments</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>Mean interval between treatments (weeks)*</td>
<td>11.3 ± 0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Monthly Injections</th>
<th>#Bimonthly Injections</th>
<th>TEP/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean # of treatments</td>
<td>13</td>
<td>8</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Abbreviations: No., number; ETDRS, Early Treatment Diabetic Retinopathy Study letter score; CST, central subfield thickness; TEP/M, treat and extend pause/monitor. Values displayed as mean ± standard error of the mean. *Mean interval between treatments were determined by limiting any interval with a value beyond 26 weeks to a maximum value of 26 weeks. #Following mandatory 3 monthly injections at initiation of treatment.
Table 3. Outcome measurements for TEP/M patients at 24 months.

<table>
<thead>
<tr>
<th>Characteristic at 24 Months</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Eyes</td>
<td>65</td>
</tr>
<tr>
<td>Mean # of treatments</td>
<td>12.2 ± 5.1</td>
</tr>
<tr>
<td>Mean interval between treatments (weeks)*</td>
<td>14.6 ± 1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Monthly Injections</th>
<th>#Bimonthly Injections</th>
<th>TEP/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean # of treatments</td>
<td>25</td>
<td>14</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Abbreviations: TEP/M, treat and extend pause/monitor. Values displayed as mean ± standard error of the mean. *Mean interval between treatments were determined by limiting any interval with a value beyond 26 weeks to a maximum value of 26 weeks. #Following mandatory 3 monthly injections at initiation of treatment.
<table>
<thead>
<tr>
<th>Time Point</th>
<th>Characteristics</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 months</td>
<td>Number of eyes</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Total eyes successfully weaned off treatment by 12 months - % (no.)</td>
<td>31% (32)</td>
</tr>
<tr>
<td>24 months</td>
<td>Number of eyes</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Total eyes successfully weaned off treatment by 24 months - % (no.)</td>
<td>38% (25)</td>
</tr>
</tbody>
</table>

Abbreviation: No., number; TEP/M, treat and extend pause/monitor.
Table 5. Characteristics of a second independent cohort of nvAMD patients receiving anti-VEGF therapy using TEP/M at a separate tertiary hospital-based clinical site who were eligible for study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Screened Patients</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total eyes (Patients)</td>
<td>32 (31)</td>
<td>20 (19)</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>75.7 ± 1.7</td>
<td>78.2 ± 1.8</td>
</tr>
<tr>
<td>Females – % (no.)</td>
<td>69% (22)</td>
<td>70% (14)</td>
</tr>
<tr>
<td>Pseudophakic - % (no.)</td>
<td>69% (22)</td>
<td>65% (13)</td>
</tr>
<tr>
<td>Presenting vision (ETDRS letters)</td>
<td></td>
<td>67 ± 3</td>
</tr>
<tr>
<td>Presenting CST (µm)</td>
<td></td>
<td>309.8 ± 19.8</td>
</tr>
<tr>
<td>Total eyes successfully weaned from treatment by 12 months in second cohort – % (no.)</td>
<td>30% (6)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: No., number; ETDRS, Early Treatment Diabetic Retinopathy Study letter score; CST, central subfield thickness; TEP/M, treat and extend pause/monitor. Values displayed as mean ± standard error of the mean.
Table 6. Proportion of patients in TEP/M protocol with no fluid on OCT from 0 to 6 months.

<table>
<thead>
<tr>
<th>Month</th>
<th>Non-weaned n = 84</th>
<th>Weaned n = 38</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2% (1)</td>
<td>0.0% (0)</td>
<td>0.497</td>
</tr>
<tr>
<td>1</td>
<td>29.8% (25)</td>
<td>63.2% (24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>46.2% (39)</td>
<td>84.2% (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>48.8% (41)</td>
<td>84.2% (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>48.8% (41)</td>
<td>94.7% (36)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: No., number; TEP/M, treat and extend pause/monitor. Values displayed as mean ± standard error of the mean. Statistical analysis was performed using Chi-square and Mann-Whitney test. Data in bold are statistically significant.